IMMUNOMODULATORY ACTIVITY OF *LACTOBACILLUS SPOROGENES*

V BABAR [1], R THOMAS [1], M. BHASKAR [2] *

[1] Department of Clinical Pharmacy and [2] Department of Pharmacology, Shobhaben Pratapbhai Patel School of Pharmacy and Technology Management, SVKM's NMIMS, V.L. Mehta Road, Ville Parle (West), Mumbai-400056
[2] SPP-SPTM, SVKM's NMIMS, Vile Parle (West), Mumbai-400056, Maharashtra.

ABSTRACT

The present study was undertaken to explore the immunomodulatory activity of probiotic strain *Lactobacillus sporogenes* on total leukocyte count, differential leukocyte count, neutrophil adhesion test, humoral response to BCG vaccine, delayed-type hypersensitivity and phagocytic activity.

Pre-treatment with probiotic evoked a significant increase in total leukocyte count and per-cent neutrophil and lymphocyte increase in differential leukocyte count. The probiotic strain produced a significant increase in neutrophil adhesion to nylon fibres. The augmentation of humoral immune response to BCG vaccine by *Lactobacillus sporogenes* (1 gm powder contains not less than 150 million spores) is evidenced by significant increase in antibody titres in rats. Oral administration of probiotic spores in rat, for a period of 28 days, significantly potentiated the delayed-type hypersensitivity reaction induced by BCG vaccine. The strain was also evaluated for *in vivo* phagocytic activity by carbon clearance assay in mice wherein, significant increase in the phagocytic index was observed.

Thus the study stated that the probiotic strain *Lactobacillus sporogenes* shows a significant stimulation of the cell mediated immunity and humoral immunity.

Key words: neutrophil, delayed type hypersensitivity, *Lactobacillus sporogenes*, immunity, probiotic.

INTRODUCTION

The immune system of mammals includes a complex array of cells and molecules, which interact to provide protection from challenge by pathogenic microbes (bacteria, viruses, parasites). Antigens are substances that induce an immune response, and are often components of invading microbes.

Probiotics are defined as the viable microorganisms that exhibit a beneficial effect on the health of the host by improving its intestinal microbial balance [1-2]. Since the beginning of the previous century, various health promoting effects of human consumption of probiotic bacteria have been described [3-4]. Numerous experiments have indicated that (changes in) the intestinal microbiota can cause immunomodulation, both at the intestinal and the systemic level [5-6]. Especially lactic acid bacteria (LAB), that are part of the human commensal microbiota and that have a long history of use in food products, have been studied widely. The ability of several LAB strains to modulate host innate as well as acquired immune responses, has been demonstrated in many in vitro experiments and animal models. Besides stimulation of antibody production [7] and macrophage activity [8], functional effects like inhibition of inflammation [9-10], intestinal infections [11], allergic disease symptoms [12-13] and autoimmune.

Few probiotic strains are evaluated and reported for immunomodulatory activity. *Lactobacillus acidophilus* has been reported to stimulate a non-specific immune response in germ-free Swiss mice as indicated by the stimulation of the host mononuclear phagocytic activity. The oral administration of *Lactobacillus casei* strain in mice stimulated type 1 helper T (Th1) cells, activated the cellular immune system and inhibited incidence of tumors and IgE (Immunoglobulin E) production [14]. *Lactobacillus sporogenes* binds with other beneficial microorganisms in the human gut and aids in digestion of food, regulation of metabolism and production of vitamins. *Lactobacillus sporogenes* maintains the effective functioning of the intestines and promotes healthy intestinal function by producing lactic acid [15]. The bacterium is effective against a range of gastrointestinal disorders

*Corresponding author:
Email: manju.bhaskar@nmims.edu

(including diarrhea, inflammatory bowel disease, irritable bowel syndrome and ulcers) and improves the symptoms of stomach acidity and lactose intolerance. *Lactobacillus sporogenes* maintains a healthy balance of microflora in the gut and supports the growth of similar beneficial bacteria [15]. Evidence suggests that, oral supplementation with *Lactobacillus sporogenes* for several days colonized this probiotic in the human gut flora better way than other strain [14].

The present study was therefore undertaken to explore the immunomodulatory activity of *Lactobacillus sporogenes* on cellular and humoral immune responses to the antigenic challenge by BCG vaccine and by neutrophil adhesion test, phagocytic activity and delayed –type hypersensitivity reaction.

MATERIAL & METHODS

Test Strain

SPORLAC®, a powder sachet manufactured by UNI-SANKYO Ltd was used as a source of probiotic strain *Lactobacillus sporogenes*. Each sachet of 1 gm powder contains not less than 150 million spores *Lactobacillus sporogenes* (lactic acid bacillus). During the experimentation the dose selected was 1 sachet per rat.

Animals

Adult Wistar rats (100-200g) and Swiss albino mice (18-20g) of either sex kept at the Central Animal House of Shobhaben Pratapbhai Patel School of Pharmacy & Technology Management, SVKM's NMIMS, Mumbai, India were used. The animals were housed under standard environmental conditions had free access to standard pellet diet (Nutrimix Lifesciences) and water ad libitum.

Antigenic Material

BCG vaccine was used as antigenic material. It was obtained from Serum Institute of India Limited. TUBERVAC® vial contained live freeze-dried BCG (Bacillus Calmette-Guerin Vaccine I.P.) vaccine derived from attenuated strain of *Mycobacterium bovis*. Each 1ml contains between 1×10^6 to 33×10^6 Colony Forming Units (C.F.U.). During the experimentation the dose selected was 0.1 ml of BCG vaccine i.p. or s.c. for immunization and challenge respectively.

Reagents used

Heparin, Methanol, Bovine Serum Albumin, Sodium chloride and Leishman's stain were purchased from Sigma Aldrich.

Well no	Dilution (antibody titre)
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024

Animals

Adult Wistar rats (100-200g) and Swiss albino mice (18-20g) of either sex were housed under standard environmental condition for a period of one week. They were provided with a standard diet supplied by Nutrimix Lifesciences, Pune, India and water ad libitum at central animal house. All the experimental protocols were approved by institutional animal ethics committee, Shobhaben Pratapbhai Patel School of Pharmacy & Technology Management, SVKM's NMIMS. Regd no:- CPCSEA/IAEC/SPTM/P-14/2011.

Adult Wistar rats were divided into two groups of six animals each. Group I served as control (distilled water) whereas Group II was administered with the test drug (*Lactobacillus sporogenes*:-1 sachet per rat), dissolved in distilled water and fed orally for a period of 14 days. Wistar rats were used for Total Leukocyte count, Differential leukocyte count, Neutrophil adhesion test, Delayed type hypersensitivity (DTH), Humoral antibody (HA) titre.

Swiss albino mice were also divided into two groups of six animals each. Group I served as control (distilled water) whereas Group II was administered with the test drug (*Lactobacillus sporogenes*:-1 sachet per rat), dissolved in distilled water and fed orally for a period of 14 days. These animals were used for Carbon clearance test (phagocytic activity).

Total leukocyte count (TLC Count) [16]

Before starting the experiment, blood withdrawn from retro-orbital vein was taken for baseline readings for total leukocyte count (WBC count). TLC count done by Sysmex cell counter.

Differential leukocyte count (DLC Count) [16]

Blood withdrawn from retro-orbital vein was taken for baseline readings of differential leukocyte count. A thin blood film was prepared on a clean, dry, glass slide. It was dried, fixed with methanol and stained with Leishman's stain. One drop of cedar wood oil was placed over the film. The cells were identified and entered into 100 squares. This gives the % of different types of leukocytes present in rat blood.

After initial TLC and DLC counts the control group animals received vehicle whereas test group animals were administered with test drug (*Lactobacillus sporogenes*:-1 sachet per rat), dissolved in distilled water and fed orally for a period of 14 days.

Neutrophil adhesion test [17-19]

On the 14th day after drug treatment, blood samples were collected by puncturing the retro orbital plexus into heparanized vials and analyzed for total leucocyte count (TLC) by Sysmex cell counter and differential leucocyte count (DLC) by fixing blood smears and staining with Leishman's stain. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibers for 15 min at 37 °C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample. Finally, percent neutrophil adhesion was calculated.

$$\text{Neutrophil adhesion } (\%) = \frac{NI_u - NI_t}{NI_u} \times 100$$

NI_u is the Neutrophil index of untreated blood samples and NI_t is the Neutrophil index of treated blood samples

Haemagglutinating antibody (HA) titre [16]

On 14th day after drug treatment, rats of both the groups were immunized with 0.1ml of BCG vaccine/ rat by intraperitoneal route. The day of immunization was referred to as day 0. The group II animals were administered with test drug for 14 more days whereas group I received water for 14 days. Blood samples were collected from each rat on day 15 for HA titre. Two fold serial dilution was performed of 1 volume of serum sample with 1 volume of 0.1% Bovine Serum Albumin (BSA) in saline. The tubes were marked from I to X.

One volume of 0.1% BCG vaccine in BSA in saline was added to 10 tubes. 1 ml from tube no one was added to second tube. The tubes were mixed thoroughly and the same procedure continued up to tube number X. They were allowed to settle down at room temperature for 60-90 minutes until control tube showed unequivocally negative pattern (a small button formation). The button formation was observed. The well which is previous to the well showing button formation was considered as Antibody titre.

Delayed type hypersensitivity [17]

Six animals per group (control and treated) were immunized by intraperitoneal administration of 0.1 ml of BCG vaccine per rat and challenged by subcutaneous administration of 0.1 ml of BCG vaccine per rat into right hind foot pad on day +14.The test compound was administered orally from day − 14 until day +13.DTH response was measured at 24, 48, 72 h after challenge on days +14, +15 and +16. It was expressed as mean percent increase in paw volume by using vernier caliper.

In vivo phagocytic activity by carbon clearance assay [20]

The control group received vehicle whereas the treatment groups were administered with test drug orally daily for 20 days. Colloidal carbon solution, Rotring ink® (Hamburg, Germany) was diluted with normal saline (1:8), and injected (0.01 ml/g body weight) was via tail vein to each mouse 24 h after last dose. Blood samples were drawn from retro-orbital plexus under ether anesthesia at 2 and 15 min after injection. Blood (25µl) was mixed with 0.1 % sodium carbonate (2 ml) for the lysis of erythrocytes OD was recorded at 660 nm. The phagocytic index (K) was calculated by using following equation:

K= (ln OD1-ln OD2) / (T2-T1), where OD1 and OD2 are the optical densities at times T1 and T2, respectively [21].

Statistical Analysis

All data were expressed as mean ± S.D. and analyzed statistically by using Dunnett's t test. A difference was considered significant at $P < 0.05$ and highly significant at $P<0.01$, as compared to control.

RESULTS

Effect of pre-treatment with *Lactobacillus sporogenes* on Total Leukocyte Count

Oral administration of *Lactobacillus sporogenes* for 14 days showed that there was a highly significant (P<0.01) increase in mean total leukocyte count as compared to control group animals (Fig. 1).

Effect of pre-treatment with *Lactobacillus sporogenes* on Differential Leukocyte Count

Oral administration of *Lactobacillus sporogenes* for 14 days showed that the animals treated with *Lactobacillus sporogenes* showed a significant (P<0.05) increase in mean % of neutrophils and lymphocytes as compared to control (Table 1).

Effect of pre-treatment with *Lactobacillus sporogenes* on Neutrophil Adhesion Test

Pretreatment with *Lactobacillus sporogenes* evoked a significant (P < 0.05) increase in the *in vitro* neutrophil adhesion to nylon fibres, which correlates with the increase in percent neutrophils (Table 2).

Effect of pre-treatment with *Lactobacillus sporogenes* on Haemagglutinating antibody (HA) titre

The HA titre was used to assess humoral immune response. A highly significant (P<0.01) increase in antibody titre was observed in mice treated with *Lactobacillus sporogenes*. The augmentation of the humoral immune response to BCG by *Lactobacillus sporogenes* is evidenced by an increase in the antibody titres in the blood of rats (Table 3).

Effect of pre-treatment with *Lactobacillus sporogenes* on Delayed type hypersensitivity

The cell-mediated immune response was assessed by DTH reaction, i.e. foot pad reaction. The test drug, *Lactobacillus sporogenes* produced a highly significant (P<0.01), increase in DTH reactivity in rats. Increase in DTH reaction in mice in response to T cell dependent antigen revealed the stimulatory effect of *Lactobacillus sporogenes* on T cells (Table 4).

Effect of pre-treatment with *Lactobacillus sporogenes* on Phagocytic response

Macrophages accomplish nonspecific immune function through phagocytosis. *In vivo* phagocytic activity of *Lactobacillus sporogenes* was determined

Table 1: Effect of pre-treatment with *Lactobacillus sporogenes* on Differential leukocyte count

Differential leukocytes	Group I	Group II
% Neutrophils	48.5 ± 5.39	67.8 ± 2.95*
% Lymphocytes	35.83 ± 5.67	53.4 ± 2.19*
% Monocytes	7.67 ± 1.36	2.8 ± 1.30
% Basophils	3.17 ± 0.98	2.4 ± 0.89
% Eosinophils	4.84 ± 1.16	3.6 ± 1.51

Group I: control (without any drug treatment); Group II: *Lactobacillus sporogenes* treated group Values are mean ± S.D. *P < 0.05 are significantly different from the values of the control group.

Table 2: Effect of pre-treatment with *Lactobacillus sporogenes* on Neutrophil Adhesion Test

Groups	Neutrophil index		Neutrophil adhesion (%)
	Untreated	Treated	
Group I	397.52±118.35	220.74±56.17	44.47
Group II	699.86±376.57	330.72±139.05	52.74*

Group I: control (without any drug treatment); Group II: *Lactobacillus sporogenes* treated group Values are mean ± S.D. *P < 0.05 are significantly different from the values of the control group.

Table 3: Effect of pre-treatment with *Lactobacillus sporogenes* on Haemagglutinating antibody (HA) titre

Groups	Antibody titre
Group I	21.33±9.23
Group II	85.33±36.95**

Group I: control (without any drug treatment); Group II: *Lactobacillus sporogenes* treated group Values are mean ± S.D. **P < 0.01 are highly significantly different from the values of the control group.

Table 4: Effect of pre-treatment with *Lactobacillus sporogenes* on Delayed type hypersensitivity

Groups	Foot pad thickness (cm)		
	24hr	48hr	72hr
Group I	0.638±0.05	0.632±0.03	0.624±0.03
Group II	0.505±0.068 **	0.477±0.059 **	0.456±0.057 **

Group I: control (without any drug treatment); Group II: *Lactobacillus sporogenes* treated group. Values are mean ± S.D. **P < 0.01 are highly significantly different from the values of the control group.

Table 5: Effect of pre-treatment with *Lactobacillus sporogenes* on Phagocytic response

Groups	Phagocytic index
Group I	0.062±0.004
Group II	0.088±0.005**

Group I: control (without any drug treatment); Group II: *Lactobacillus sporogenes* treated group. Values are mean ± S.D. **P < 0.01 are highly significantly different from the values of the control group.

by the carbon clearance assay in mice. The results of this assay are presented in Table 5. The phagocytic index (K) for *Lactobacillus sporogenes* was highly significant (P<0.01) as compared to control group.

DISCUSSION

Immunomodulation through stimulation or suppression may help in maintaining a disease-free state. Immunomodulatory agents obtained from plant and animal origin enhances immune responsiveness of an organism against a pathogen by activating the immune system. Probiotic consumption is reported to exert a myriad of beneficial effects including enhanced immune response, balancing of colonic microbiota, vaccine adjuvant effects, reduction of fecal enzymes implicated in cancer initiation, treatment of diarrhea associated with travel and antibiotic therapy [1-2]. There is a growing interest in identifying probiotics as immunomodulators ever since their possible use

in modern medicine has been suggested [2]. The results obtained in the present study indicate that *Lactobacillus sporogenes* is a potent immunostimulant, stimulating both the specific and non-specific immune mechanisms.

The neutrophil, an end cell unable to divide and with limited capacity for protein synthesis is, nevertheless, capable of a wide range of responses, in particular chemotaxis, phagocytosis, exo-cytosis and both intracellular and extracellular killing [20]. In the present study, pre-treatment with evoked a significant increase in percent neutrophils. This may help in increasing immunity of body against microbial infections [17].

Antibody molecules, a product of B lymphocytes and plasma cells, are central to humoral immune responses, IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, neutralization of toxins, etc. [21]. The augmentation of the humoral immune response to BCG by *Lactobacillus sporogenes*, as evidenced by increase in the antibody titre in rats (Table 3) indicated the enhanced responsiveness of T and B lymphocyte subsets, involved in the antibody synthesis [17].

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). CMI responses are critical to defense against infectious organisms, and delayed-type hypersensitivity reactions [21]. Therefore, increase in DTH reaction in rats in response to T cell dependent antigen revealed the stimulatory effect of the probiotic strain on T cells (Table 4).

Phagocytosis is the process by which certain body cells, collectively known as phagocytes, ingest and removes microorganisms, inorganic particles and tissue debris [21]. *Lactobacillus sporogenes*

appeared to enhance the phagocytic function by

REFERENCES

Fig. 1: Effect of pre-treatment with *Lactobacillus sporogenes* on Total leuckocyte count
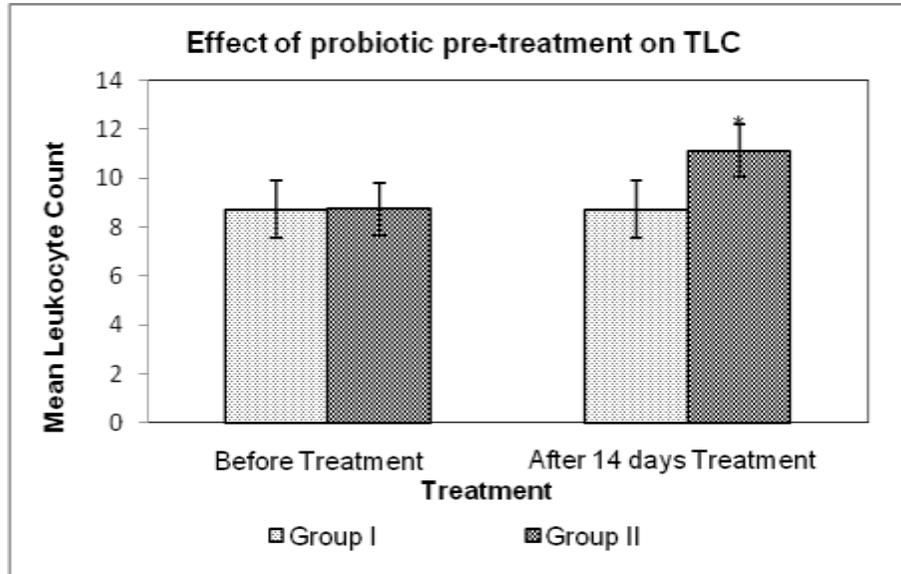
Group I: control (without any drug treatment); Group II: *Lactobacillus sporogenes* treated group. Values are mean ± S.D. **P < 0.01 are highly significantly different from the values of the control group.

exhibiting a dose related increase in clearance rate of carbon by the cells of the reticulo-endothelium system (Table 5).

The present investigation suggests that the probiotic strain, *Lactobacillus sporogenes* may stimulate both cellular and humoral immune responses.

Conflict of interest

The authors declare that they have no conflict of interest.

1. Nicole, C., Dan, D, 2010. Probiotics-Identification and ways of action- innovative. Romanian Food Biotechnology 6: 1-11.

2. Kaur, I., Chopra, K., Sainia, A, 2002. Probiotics: potential pharmaceutical applications. European Journal of Pharmaceutical Sciences 15: 1-9.

3. Gill, H. S., Guarner, F, 2004. Probiotics and human health : a clinical perspective. Postgraduate Medical Journal 80: 516 –526.

4. Kumar, S.G., Singh, S.K., Goyal, P., Dilbaghi, N., Mishra, D.N., 2005. Beneficial effects of probiotics and prebiotics on human health. Pharmazie 60: 163–171.

5. de Vrese, M, Schrezenmeir, J, 2002. Probiotics and non-intestinal infectious conditions. The British Journal of Nutrition 88 (Suppl 1): S59–S66.

6. Noverr, M.C., Huffnagle, G.B, 2004. Does the microbiota regulate immune responses outside the gut? Trends in Microbiology 12: 562 –568.

7. Alvarez-Olmos, M.I., Oberhelman, R.A., 2001. Probiotic agents and infectious diseases: a modern perspective on a traditional therapy. Clinical Infectious Diseases 32: 1567–1576.

8. Schiffrin, E.J., Rochat, F., Link-Amster, H., Aeschlimann, J.M., Donnet-Hughes, A., 1995. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. Journal of Dairy Science 78: 491– 497.

9. Hart, A.L., Stagg, A.J., Kamm, M.A., 2003. Use of probiotics in the treatment of inflammatory bowel disease. Journal of Clinical Gastroenterology 36:111–119.

10. Chapat, L., Chemin, K., Dubois, B., Bourdet-Sicard, R., Kaiserlian, D., 2004. *Lactobacillus casei* reduces CD8+T cell -med iated skin inf lammatio n. European Journal of Immunology 34: 2520–2528.

11. Marteau, P. R., de Vrese, M.., Cellier, C.J., Schrezenmeir, J., 2001. Protection from gastrointestinal diseases with the use of probiotics. The American Journal of Clinical Nutrition 73: 430S–436S.

12. Isolauri, E., Sutas, Y., Kankaanpaa, P., Arvilommi, H., Salminen, S. 2001. Probiotics: effects on immunity. The American Journal of Clinical Nutrition 73: 444S–450S.

13. Kalliomaki, M., Isolauri, E., 2003. Role of intestinal flora in the development of allergy. Current Opinion in Allergy and Clinical Immunology 3: 15–20.

14. Adami, A., Cavazzoni, V., 1993. Biomass production, preservation and characteristics of a strain of Bacillus coagulans as probiotic. Microbiol Aliments Nutr 11: 9-100.

15. Delcenserie, V., Martel, D., Lamoureux, M., Roy, D., 2009. Immunomodulatory Effects of Probiotics in the Intestinal Tract. Current Issues in Mol. Biol 10: 37-54.

16. Singh, M., Ahirwar, J., Muthal, N., 2011. Evaluation of immunomodulatory activity of aqueous extract of *Ficus bengalensis* aerial roots in Wistar rats. Asian Journal of Pharmaceutical and Clinical Research 4: 82-86.

17. Ghule, B., Murugananthan, G., Nakhat, P., Yeole, P., 2006. Immunostimulant effects of *Capparis zeylanica* Linn. leaves. Journal of Ethnopharmacology 108: 311–315.

18. Chakraborthy, G., 2009. Evaluation of Immunomodulatory activity of *Aesculus indica*. International Journal of PharmTech Research 1: 132-134.

19. Yan, Y., Wanshun, L., Baoqin, H., Changhong, W., Chenwei, F., Bing, L., Liehuan, L., 2007. The antioxidative and immunostimulating properties of D-glucosamine. International Immunopharmacology 7: 29–35.

20. Dale, M., Foreman, J., 1989. Textbook of Immunopharmacology. Black-well, London 52–54.

21. Miller, L.E., 1991. In: Ludke, H.R., Peacock, J.E., Tomar, R.H. (Eds.), Manual of Laboratory Immunology. Lea and Febiger, London.